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Genetic Immunization

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ABSTRACT

Genetic immunization, the latest addition to the field of vaccinology, has shown, in a number of animal models, to be an efficacious approach to induce protective immunity to infectious diseases. The advantages of DNA vaccines are their ease of construction, the low expanse of mass production, their high temperature stability, and their ability to induce a full spectrum of exceptionally long-lasting immune responses including cytolytic T cells. Their potential disadvantages are putative safety issues such as integration into the host cell genome. The slow development of the immune response to genetic immunization will make these vaccines unsuitable for treatment of some infectious diseases such as postexposure vaccination to rabies virus, where a rapid immune response is warranted. Although only time will tell if genetic immunization provides a viable alternative for human immunization, in the meantime this approach provides immunologists with a powerful tool to gain further insight in the mechanisms that drive primary immune responses.

INTRODUCTION

Direct *in situ* gene transfer by inoculation of plasmid vectors was initially developed for postnatal gene therapy which aims at replacing missing or faulty genes (1). During these studies intramuscularly injected circular DNA was found to be taken up by muscle cells without requiring any of the special delivery systems needed for efficacious *in vitro* transfection of cell lines (2–4). The inoculated DNA stayed episomally for a prolonged period of time causing expression of the vector-encoded proteins that in the initial studies were reporter proteins such as chloramphenicol acetyltransferase, luciferase, and β-galactosidase. Two years after these findings another group made the quantum leap and upon inoculation of mice with a plasmid vector expressing a foreign protein demonstrated stimulation of an antibody response that started the era of DNA vaccines also called genetic immunization (5). The later term was coined by S.A. Johnston, who rightfully felt that the name DNA vaccine implied the intention to induce immunity to DNA (S.A. Johnston, personal communication). Now, only 3 years later, genetic immunization that not too long ago was unkindly referred to as the "biological equivalent of cold fusion" (6) is coming of age not just as a potential vaccine approach but also as a tool to study basic immune responses. Genetic immunization has a number of advantages over traditional vaccines. Plasmid vectors can be constructed rapidly and with comparative ease. Cloning a gene into a commercially available vector with a suitable multicloning site and testing expression of the protein by transient transfection can be achieved within a week. Cloning the same gene into a vaccinia recombinant virus takes well over a month. Growing a large batch of plasmid takes,

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including purification and quantitation, 2–3 days; generating a large batch of recombinant vaccinia virus takes, including purification by gradient centrifugation and titration by a plaque assay, at least 2 weeks. The generation of other viral recombinants such as E1-deleted adenoviral recombinants is even more time consuming. Needless to say that the expense of producing recombinant viruses is several magnitudes higher than that of generating expression vectors. Circular DNA is stable, can be boiled, precipitated with ethanol, and shipped to collaborators in an envelope, procedures that would fare poorly with a recombinant virus. If plasmid vectors will ever be used for mass vaccinations their high temperature stability will be of extreme value, especially for developing countries where cold chains are difficult to maintain.

The technical ease of generating expression vectors is one of the reasons why the field of genetic immunization has progressed explosively in the past 3 years. In this review we will summarize the most pertinent features of published data and discuss some of the issues that remain to be investigated.

Experimental models of genetic immunization. The first report of induction of a B cell response upon genetic immunization of mice using the human growth factor as the antigen (5) has been confirmed in a variety of other systems. Using an expression vector for the influenza virus nucleoprotein, stimulation of cytolytic T cells was demonstrated upon genetic immunization (7). The same report furthermore showed induction of protective immunity to challenge with the virus. Subsequent studies described stimulation of both humoral and cell-mediated immunity upon inoculation of vectors expressing a wide variety of antigens such as the hemagglutinin of influenza virus (8), the envelop protein of human immunodeficiency virus (HIV) 1 (9), the glycoprotein of rabies virus (10), the glycoprotein B of herpes simplex virus (11), the core protein of hepatitis C virus (12), the hepatitis B virus surface antigen (13), the circumsporozoite protein of *Plasmodium yoelii* (14), and the major surface protein of *Leishmania major* (15). Regardless of the antigen or the technical approach of genetic immunization, the reports were rather similar by describing stimulation of antibodies and cytolytic T cells and, where the appropriate animal model was available, induction of protection to challenge with the pathogen. The initial studies conducted in mice were rapidly and successfully expanded to other species such as ferrets (16), rabbits (17), primates (18), chicken (8), and cattle (19). Most publications reported success; failures tend to get rejected and thus remain unpublished. We found in the rabies virus system that although the viral glycoprotein, a surface protein that stimulates in context of the virion or a vaccinia rabies glycoprotein recombinant virus T helper cells, neutralizing antibodies, and cytolytic T cells, could readily induce an immune response upon inoculation of an appropriate expression vector, the nucleoprotein of rabies virus, which, if presented to the immune system in a different form elicits a potent T helper and B cell response but no cytolytic T cells, failed to stimulate a measurable response upon genetic immunization. This shows that although any antigens can be delivered by genetic immunization, some proteins upon expression by plasmid vectors remain immunologically silent. The principles that govern success versus failure of genetic immunization with regard to each individual protein remain to be elucidated. Cellular localization of the foreign protein might play a role; the nucleoprotein of rabies virus remains intracellular, while the glycoprotein is expressed on the cell membrane. Other intracellularly retained antigens such as the nucleoprotein of influenza A virus (7) have been used successfully as genetic vaccines. The influenza virus nucleoprotein as opposed to the nucleoprotein of rabies virus stimulates a cytolytic T cell response. Correlating the two findings and predicting that the potential ability of a protein to induce cytolytic T cells is a prerequisite for an intracellular antigen to be immunogenic upon genetic immunization are tempting, but require further confirmation.

Conditions that affect the efficacy of genetic immunization. Transfer of plasmid DNA *in situ* was initially demonstrated upon intramuscular inoculation (2). In the meantime, successful immunization has been demonstrated by a number of other routes such as upon intradermal (5,20) and intravenous injections and upon application to mucosal membranes (8). In several studies the fate of the DNA upon inoculation was investigated (2–5). Transfected cells could be demonstrated only close to the site of injection. In one study DNA was inoculated into the tip of the ear of mice. Within minutes the ear was surgically removed. Nevertheless, the mice developed antibodies to the vector encoded protein (S.A. Johnston, personal communication), which contradicts the finding that the DNA stays strictly locally at the site of inoculation. A minute amount of DNA, below the level of detectability that either remained extracellularly or was taken up by migratory cells, might reach the lymph nodes where vector-encoded antigens upon processing and presentation by dendritic cells could initiate an immune response.

Some studies have used saline to dilute the DNA prior to injection (13). We have used distilled water (10); others have used hypertonic solutions based on sucrose (4). Drugs causing muscle damage such as buvocain (9) or cardiotoxin (13) were shown by some investigators to improve the efficacy of genetic vaccines. Immune responses have been induced upon injection of DNA with a syringe (10,13), upon intradermal inoculation of DNA coated to gold particles using a gene gun (5) or upon intramuscular injection using a pneumatic gun (21). All of these approaches resulted in an immune response, and there is no clear indication that one method is truly different or superior to the other. Injecting the DNA in a larger rather than a smaller volume seems to give better results. Gene gun delivery of DNA coated to gold particles allows a reduction of the amount of DNA; while a good immune response to DNA delivered by a syringe requires anywhere from 10 to 100 μg of vector, particle bombardment allows a reduction down to the nanogram range.

The level of expression of the vector-encoded protein presumably affects the magnitude of the immune response. Level of expression depends on a variety of variables such as stability of the RNA, copy number of vector per cell, presence of introns in the expression vector, and spacing between the regulatory element and the gene. One of the most important factors is the type of promoter that drives expression of the protein. Most genetic vaccines rely on viral promoters that function in a wide spectrum of cells. The early promoter of cytomegalovirus (CMV) and the Rous sarcoma virus were both shown to work well in genetic vaccines. The simian virus (SV) 40 promoter that, depending on the cell type, causes only 2–5% of the expression initiated by the CMV promoter was found by some investigators to be inappropriate as a regulatory element in genetic vaccines (4). Nevertheless, for genetic vaccines encoding the rabies virus glycoprotein, vectors based on the SV40 promoter induced a good immune response (4). Replacing the SV40 promoter with the more potent CMV promoter, leaving all other parameters of the plasmid intact, did not improve the immune response to the rabies virus glycoprotein (22). *In vitro* expression of the glycoprotein upon stable or transient transfection with the vector based on the SV40 promoter could readily be demonstrated. Vectors that contained the CMV promoter induced only a very transient expression of the rabies virus glycoprotein, which most likely reflects toxicity of this protein upon overexpression (22). In addition to viral promoters, mammalian promoters such as the β-actin promoter (5) and the MHC class I promoter (Ertl et al., unpublished data) have been used successfully in genetic vaccines. To summarize, with regard to the regulatory unit driving expression of the foreign protein, strong promoters with broad tissue specificity are generally best, although for proteins that have a toxic effect upon overexpression, weaker promoters leading to lower but sustained expression might be advantageous.

The immune response to genetic immunization. As already mentioned, genetic immunization results in a full spectrum of immune responses to the expressed protein including antibodies, T helper cells, and cytolytic T cells. Although we initially published the T helper cell response to be of the Th1 type characterized by secretion of interleukin (IL)-2 in absence of IL-4 (10), further studies such as isotype mapping of the antibody response indicated that at least in the rabies virus system the response is a mixed Th0 like response that results in both IgG_1, IgG_{2a}, and IgG_{2b} type antibodies. Interferon-γ (IFN-γ), a cytokine that is typical for a mature Th1 response, was not secreted at high levels by T helper cells induced to the rabies virus glycoprotein upon genetic immunization.

The kinetic of the immune response to genetic immunization was clearly different from that observed after traditional vaccines. In most systems animals inoculated with a plasmid vector developed an immune response rather slowly. Antibodies in our system based on the rabies virus glycoprotein could not be demonstrated until 3–5 weeks after vaccination. They reached peak titers approximately 10 weeks after a single immunization and then remained at a constant level for at least 11 months (the latest time point we have tested thus far, 22). Other groups obtained comparable results; a slowly increasing immune response that then remained at plateau levels for the lifespan of the mouse. Booster immunizations had in general little effect on the response. Initial claims that repeated immunizations given in 2 or 3 week intervals after priming did have a booster effect were most likely a reflection of the slow on-rate of the immune response to genetic vaccines (10). Although in the majority of systems the immune response to genetic immunization was exceptionally long lasting, in some cases a rapid decline of antibody titers could be observed. One example was a genetic vaccine to the gp120 of HIV-1 that resulted in a transient antibody response that could not be sustained by booster inoculations (23). Whether this reflects tolerization to this protein remains to

be investigated. We found that coimmunization of mice with a vector expressing the rabies virus glycoprotein and an additional vector expressing mouse granulocyte macrophage–colony–stimulating factor (GM-CSF) caused an increase of the initial immune antibody response followed by a decline (24). These results that are described in more depth below lead us to assume that a potent immune response to the vector-encoded protein might result in rapid elimination of the transfected muscle cells, thus eliminating the antigen required for the sustained immune response.

Mechanisms of genetic immunization. The precise mechanism of initiation of an immune response upon genetic immunization remains to be elucidated. Most studies to date have used intramuscular inoculation of plasmid DNA. In several of these studies expression of proteins such as β-galactosidase (2) or hepatitis B virus surface antigen (25) was demonstrated in myoblasts and myotubes leading to the suggestion that muscle cells process and present the antigen and stimulate a primary immune response. Muscle cells express only low levels of MHC class I antigen and they lack MHC class II expression (26). IFN-γ was shown *in vitro* to upregulate MHC class I expression and to induce MHC class II expression on a number of cells including muscle cell lines (26). Transfection of muscle cells using a syringe causes minimal damage and does not result in an extensive histologically visible inflammatory reaction that might provide IFN-γ, which then, in turn, might locally upregulate MHC expression. Coimmunization of mice with an antigen-expressing vector and a plasmid expressing IFN-γ did not enhance the immune response (24), as would be expected if the density of MHC class I and II determinants on the transfected muscle cells was a limiting factor in initiating the immune response to genetic immunization. To the contrary, in most of our experiments, IFN-γ caused a slight but significant decrease in both the B and T helper cell response to the rabies virus glycoprotein encoded by a plasmid vector (24). Furthermore, induction of a primary T cell-mediated immune response as a rule requires presentation of the antigen by professional antigen-presenting cells, such as dendritic cells, that in addition to MHC class I and II determinants carry so-called costimulatory signals (27–29) such as B7.1 or B7.2 that are not expressed on most cells including muscle cells. Encounter of naive T cells with antigen presented by cells that lack such costimulatory signals leads to tolerance as has been shown conclusively for T helper cells. The requirements for stimulation of virgin cytolytic T cells are less well defined, but are assumed to follow a similar pathway. A recent report described induction of a primary cytolytic T cell response upon immunization of mice with fibroblasts transfected with a vector encoding an antigen of lymphocytic choriomeningitis virus (30). In F_1 mice, the response was restricted exclusively to the MHC elements present on the transfected cell lines. The authors interpreted these results as evidence that activation of primary cytolytic T cells does not require costimulatory signals. We had demonstrated earlier that an L929 cell line transfected with a vector expressing the rabies virus glycoprotein stimulated in mice a potent T helper and B cell response and hence protection to challenge with a virulent strain of the virus (31). Our interpretation was that the antigen was reprocessed and presented by professional antigen-presenting cells. Assuming for the time being that cytolytic T cells can be induced by transfected fibroblasts or muscle cells, their sustained activation requires IL-2. In absence of T helper cells their response should decline rapidly, which is in contrast to what has been observed upon genetic immunization.

Dendritic cells, considered the primary antigen-presenting cells, are present throughout most tissues (32). Upon encounter of antigen they become activated and migrate to the local lymphatic organs, where they initiate an immune response. Activated effector T cells then return to the sites of antigenic infestation and start removing the antigen. Once the antigen has been eliminated the acute phase of the immune response declines and the effector T cells undergo apoptosis. B cells remain activated at low levels by antigen released from so-called ICOSOMs present on follicular dendritic cells in lymph nodes (33,34). If the antigen has to be presented by dendritic cells upon genetic immunization, the question arises how dendritic cells acquire the antigen. The initial inoculation might lead to limited muscle cell damage. Dying cells are unable to synthesize vector-encoded proteins and are thus an unlikely source of antigen. Some antigens can be secreted by the transfected muscle cells; others stay intracellularly. The glycoprotein of rabies virus remains firmly anchored to the cell membrane; our attempt to demonstrate secretion of this protein from transfected cell lines in amounts sufficient to trigger activation of T helper cell clones were unsuccessful. We tested if forcing secretion of the rabies virus glycoprotein by inserting a stop codon just upstream of the transmembrane domain improved the immunogenicity of vector-encoded rabies virus glycoprotein. The T helper cell response to the secreted form of the protein was comparable in magnitude to that of the mem-

brane bound protein (35), indicating that secretion was not required for appropriated presentation of the antigen. Cytolytic T cells might be activated initially by antigen expressed by transfected muscle cells leading to their death, thus causing release of vector-encoded antigen. The antigen might then be taken up, processed, and presented to T helper cells by professional antigen-presenting cells. Although this mechanism cannot be ruled out at the moment, kinetic studies indicated that activation of T helper cells precedes stimulation of cytolytic T cells in this system (10). In our mind the most likely pathway for initiation of an immune response by genetic immunization is the direct transfection of professional antigen-presenting cells. The number of antigen-presenting dendritic cells needed to initiate an immune response is minute, and lack of histological evidence for direct transfection of dendritic cells might reflect either that their number is below the level of easy detectability or, alternatively, that they rapidly leave the site of inoculation and migrate to the draining lymph nodes. This interpretation is compatible with S.A. Johnston's finding that surgical removal of the site of DNA inoculation failed to abrogate the immune response and with our data showing that GM-CSF, a cytokine that activates dendritic cells, improved the T helper cell response to genetic immunization.

Another issue that requires further thought and investigation is the unusual kinetic of the immune response. The slow on-rate of the immune response that in our system requires at least 2–3 weeks until serum antibodies become detectable, not reaching peak titers until 10 weeks after immunization, might simply reflect that only a small amount of antigen is presented in an appropriate form to the immune system. Although in some systems a significant number of muscle cells was shown to express antigen, their lack of MHC class II expression renders them unrecognizable for T helper cells needed for the promotion of a mature B cell response and their low levels of MHC class I expression reduces their ability to efficiently present epitopes to cytolytic T cells. DNA transfection as opposed to virus infection causes no or only minimal damage to the afflicted cells, thus allowing the antigen to remain, at least in absence of an efficacious immune response, expressed for an indefinite period of time in nondividing cells. The combination of these two factors might lead to the slowly accumulating immune response.

How does a low dose of appropriately presented antigen sustain a constant immune response for the life span of a mouse? Admittedly, a mouse only lives, even under optimized condition, for about 2 years. Further studies in larger species with a longer life expectancy are thus needed to confirm that genetic immunization indeed achieves life long immunity and protection to microbial challenge. Although only limited data are available thus far comparing duration of protection upon inoculation with a traditional vaccine such as an inactivated virus or a live recombinant with that upon genetic immunization, they suggest that the vectors result in a longer lasting immune response (22,25,36,37). This is especially puzzling, for example, in our system, based on the rabies virus glycoprotein. Here the antibody response, which is the only immune response needed to limit the spread of rabies virus (38), is initially, during the early phase after vaccination, several orders of magnitude higher in animals vaccinated with either inactivated rabies virus or a vaccinia rabies virus glycoprotein recombinant. One speculative explanation might be that muscle cells by being very poor antigen-presenting cells serve as an antigen reservoir. The low levels of MHC class I expression might prevent cytolytic T cells from readily recognizing and eliminating the transfected muscle cells. Once in a while a cytolytic T cell might nevertheless encounter one of the sparse MHC class I antigens presenting a foreign peptide on a muscle cell leading to the death of this cell. The demise of a transfected cell would cause the release of antigen that, upon processing and presentation by professional antigen-presenting cells, might provide a stimulatory signal to T helper cells. The finding that antigen-expressing muscle cells could be demonstrated histologically only for a few weeks rather than a number of months speaks against this pathway. Life long immunity can generally be achieved only by a large antigenic load typically seen upon a natural virus infection. It is hard to envision that transfection of muscle cells achieves a comparable level of antigen. A viral infection is normally controlled by the immune system within 7–14 days. The longer duration of antigen expression upon genetic immunization might counterbalance the smaller antigenic load; in other words, two factors might play a role in achieving long lasting immunity, the initial antigenic load that determines the magnitude of the acute response, and, presumably, the frequency of specific memory cells and the duration of antigenic presence that might favor generation of memory cells over stimulation of effector cells, which are eliminated by programmed cell death once the antigen has disappeared.

Risks versus benefits of genetic vaccines. As already mentioned, some of the advantages of genetic vaccines are their ease of construction and purification and their high temperature stability. As opposed to vaccines based on inactivated pathogens, proteins, or peptides, plasmid vectors induce, in addition to T helper cells and antibodies, cytolytic T cells that play a major role in limiting the spread of viruses or cancer cells. Other subunit vaccines such as viral recombinants based on vaccinia or adenovirus not only carry the risks inherent to any infectious replication-competent agent but also induce a potent immune response to the vaccine carrier that might interfere with repeated use of such vaccines.

Although vaccines based on plasmid vectors carry none of the risks associated with attenuated pathogens, they might have their own set of problems. Inoculation of microgram amounts of double-stranded DNA might cause stimulation of antibodies to DNA that are associated with autoimmune diseases such as systemic lupus erythomatosus. We found that inbred mice inoculated repeatedly with large doses of plasmid vectors failed to develop detectable levels of antibodies to single- or double-stranded DNA (22). Although these data are promising, individuals prone to autoimmune disease might nevertheless develop antibodies to DNA upon genetic immunization. Further studies in appropriate animal species such as *lpr* mice are needed to address this potential risk.

Integration of vector DNA into the host cell genome causing disruption of genes required for regulation of cell growth might result in transformation and cancer. This, the most serious of all the theoretical risks of genetic immunization, could occur by retroviral insertion or homologous or random integration. Retroviral insertion is fairly efficient in dividing cells and can be avoided by choosing vectors that lack the retroviral sequences needed for insertion. The risk of homologous recombination can also be minimized. Random integration, a fairly rare event, might occur, especially if the DNA is targeted toward dividing cells such as those present in skin or mucosal membranes. Experiments conducted thus far show that the DNA remains episomally (39), and integration into the host cell genome has not been observed. Some data indicate that mammalian cells are able to compartmentalize foreign DNA such as viral DNA that penetrates the nucleus (40), a device that might have been favored by evolution to prevent random integration of unwanted genetic material and that might unintentionally benefit gene vaccinology.

Can genetic immunization result in unwanted immunological consequences such as induction of unresponsiveness due to prolonged presence of antigen on cells lacking costimulatory signals? Again although results thus far suggest that genetic immunization does not induce tolerance (22), further studies using a variety of different antigens are needed to address this potential problem.

Plasmid vectors as immunological tools. The ease of modifying genes present in vectors by, for example, site-directed mutagenesis, insertion or addition of sequences, invites their use to address basic immunological questions; the cellular localization of a protein can be changed by adding or removing signal sequences or transmembrane domains; the glycosylation or phosphorylation pattern of a protein can be modified by elimination or addition of the required anchoring sites; dominant T cell epitopes can be removed to address the potential role of so-called cryptic epitopes. We have used genetic immunization to test the role of individual cytokines in initiating a primary immune response (24). The technique we have used borders on the primitive: we mixed two plasmids, one expressing a viral antigen and the other a mouse cytokine in water, and then inoculated mice intramuscularly with this mixture. Inoculating the two vectors separately had no effect on the immune response. This presumably reflects that cytokines are strictly localized within a few cell diameters and that our inoculation method is too crude to reproducibly hit the same group of cells. The advantage of the approach is not only its simplicity but also, when compared to other available methods, its better mimicry of physiological events. Thus far the role of cytokines has been tested either *in vitro* by using secondary T cell lines or T cell clones. Needless to say that results obtained in tissue culture with already activated T cells are unlikely to reflect the *in vivo* conditions that govern activation of virgin T cells. The effect of cytokines or lack thereof has been tested by systematically applying cytokines or antibodies thereto. Cytokines act locally, and the overall side effects that are caused by flooding or depleting an entire organism might influence the observed effect. Knock-out mice lacking the ability to generate individual cytokines have shed light on the importance of cytokines; it has also shown that the immune system of a mouse is remarkably redundant and able to adjust to the complete lack of a cytokine. Genetic immunization has the advantage over these other methods of providing cytokines locally in conjunction with the antigen. The disadvantage is that we know neither the amounts of cytokines pre-

sent at the site of inoculation nor the duration of their secretion. Using plasmid vectors with inducible promoters would address the later problem while careful dose–response curves would, at least partially, address the former one.

Thus far, we have tested the effect of GM-CSF, IL-4, and IFN-γ on immunization of mice with a vector expressing the rabies virus glycoprotein under the control of the SV40 promoter. GM-CSF, as already mentioned, enhanced during the initial phase of the response both the T helper and the B cell response. GM-CSF is known to activate and recruit dendritic cells, which presumably improves presentation of the vector-encoded antigen and hence the immune response. Mice inoculated only with the antigen-expressing vector developed antibody titers that remained at the same level for at least 11 months. Mice coinoculated with GM-CSF developed substantially higher antibody titers initially, which, with time, declined well below those of the control mice, indicating that an enhanced immune response to genetic immunization might lead to more efficient removal of transfected muscle cells, thus causing a more rapid decline of the antibody titers.

IL-4, which we had assumed to drive the T helper cell toward the Th2 pathway, caused, at least in C3H/He mice, a shift in the kinetic of the immune response; the T helper and B cell response was starkly reduced during the initial phase after immunization and then increased, eventually, 5 months later, exceeding the response of control mice. The immune response in presence of IL-4 did not shift toward a Th2 type response, but remained of a Th0/Th1 type. In addition, presence of the IL-4-secreting vector clearly enhanced the cytolytic T cell response tested 5 months after immunization. We have currently no explanation for this finding. Stimulation of antibodies to IL-4 could explain the data, but our attempts to demonstrate such antibodies in the sera of mice vaccinated with the IL-4-expressing plasmid were unsuccessful (Ertl et al., unpublished data).

IFN-γ, which we had expected to improve the immune response by upregulating MHC expression on muscle cells, slightly reduced stimulation of B and T cells upon vector immunization indicating that the level of expression of MHC determinants on transfected muscle cells was not a limiting factor in intramuscular genetic immunization.

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REFERENCES

1. Williams, R.S., S.A. Johnston, M. Riedy, M.J. DeVit, S.G. McElligott, and J.C. Stanford. 1991. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc. Natl. Acad. Sci. U.S.A. 88:2726.
2. Wolff, J.A., R.W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P.L. Felgner. 1990. Direct gene transfer into mouse muscle in vivo. Science 247:1465.
3. Wolff, J.A., P. Williams, G. Acsadi, S. Jiao, A. Jani, and W. Chong. 1991. Conditions affecting direct gene transfer into rodent muscle in vivo. BioTechniques 11:474.
4. Davis, H.L., R.G. Whalen, and B.A. Demeneix. 1993. Direct gene transfer into skeletal muscle in vivo: factors affecting efficience of transfer and stability of expression. Hum. Gene Ther. 4:151.
5. Tang, D., M. DeVit, and S.A. Johnston. 1992. Genetic immunization is a simple method for eliciting an immune response. Nature (London) 356:152.
6. Fomsgaard, A. 1995. Genetic immunization—the biological equivalent of cold fusion. Ugeskr Laeger 157:4932.
7. Ulmer, J.B., J.J. Donnelly, S.E. Parker, G.H. Rhodes, P.L. Felgner, V.J. Dwarki, S.H. Gromkowski, R.R. Deck, C.M. DeWitt, A. Friedman, L.A. Hawe, K.R. Leander, D. Martinez, H.C. Perry, J.W. Shiver, D.L. Montgomery, and M.A. Liu. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259:1745.
8. Fynan, E.F., R.G. Webster, D.H. Fuller, J.R. Haynes, J.C. Santoro, and H.L. Robinson. 1993. DNA vaccines: Protective immunizations by parenteral, mucosal and gene-gun inoculations. Proc. Natl. Acad. Sci. U.S.A. 90:11478.

9. Wang, B., K.E. Ugen, V. Srikantan, M.G. Agadjanyan, K. Dang, Y. Refaeli, A.I. Sato, J. Boyer, W.V. Williams, and D.B. Weiner. 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. U.S.A. 90:4156.

10. Xiang, Z.Q., S. Spitalnik, M. Tran, W.H. Wunner, J. Cheng, and H.C.J. Ertl. 1994. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. Virology 199:132.

11. Manickan, E., R.J.D. Rouse, Z. Yu, W.S. Wire, and B.T. Rouse. 1995. Genetic immunization against herpes simplex virus. J. Immunol. 155:259.

12. Laaging, L.M., K. Meyer, D. Hoft, M. Houghton, R.B. Belshe, and R. Ray. 1995. Immune responses to plasmid DNA encoding the hepatitis C virus core protein. J. Virol. 69:5859.

13. Davis, H.L., M.-L. Michel, M. Mancini, M. Schleef, and R.G. Whalen. 1994. Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen. Vaccine 12:1503.

14. Sedegah, M., R. Hedstrom, P. Hobart, and S.L. Hoffman. 1994. Protection against malaria by immunization with plasmid DNA encoding cicumsporozoite protein. Proc. Natl. Acad. Sci. U.S.A. 91:9866.

15. Xy, D., and F.Y. Liew. 1994. Genetic vaccination against leishmaniasis. Vaccine 12:1534.

16. Webster, R.G., E.F. Fynan, J.C. Santoro, and H. Robinson. 1994. Protection of ferrets against influenza challenge with a DNA vaccine to the hemagglutinin. Vaccine 12:1495.

17. Haynes, J.R., M.D. Eienbraun, D.H. Fuller, E.F. Fynan, and H.L. Robinson. 1994. Gene-gun-mediated DNA immunization elicits humoral, cytotoxic and protective immune responses. Vaccine 94:65.

18. Wang, B., J. Boyer, K.E. Ugen, M. Merve, K. Dang, M. Agadjanyan, W.V. Williams, D.B. Werner, L. Coney, and R. Carrano. 1994. Analysis of antigen-specific immune responses induced through in vivo genetic immunization. Vaccines 94:83.

19. Cox, G., T. Zamb, and L.A. Babiuk. 1993. Bovine herpes virus 1: Immune response in mice and cattle injected with plasmid DNA. J. Virol. 67:5664.

20. Raz, E., D.A. Carson, S.E. Parker, T.B. Parr, A.M. Abai, G. Aichinger, S.H. Gromkowski, M. Singh, D. Lew, M.A. Yankaukas, S.M. Bard, and G.H. Rhodes. 1994. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. Proc. Natl. Acad. Sci. U.S.A. 91:9519.

21. Vahlsing, H.L., M.A. Yankauckas, M. Sawdey, S.H. Gromkowski, and M. Manthrope. 1994. Immunization with plasmid DNA using a pneumatic gun. J. Immunol. Methods 175:11.

22. Xiang, Z.Q., S.L. Spitalnik, J. Cheng, J. Erikson, B. Wojczyk, and H.C.J. Ertl. 1995. Immune responses to nucleic acid vaccines to rabies virus. Virology 209:269.

23. Lu, S., S.C. Santoro, D.H. Fuller, J.R. Haynes, and H.L. Robinson. 1995. Use of DNAs expressing HIV-1 env and noninfectious HIV-1 particles to raise antibody responses in mice. Virology 209:147.

24. Xiang, Z.Q., and H.C.J. Ertl. 1995. Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. Immunity 2:129.

25. Davis, H.L., M. Michel, and R.G. Whalen. 1993. DNA-based immunization for hepatitis B induces continuous secretion of antigen and high levels of circulating antibody. Hum. Mol. Genet. 2:1847.

26. Hohfeld, R., and A.G. Engel. 1994. The immunobiology of muscle. Immunol. Today 15:269.

27. Bretscher, P., and M. Cohn. 1970. A theory of self non self discrimination: Paralysis and induction involve the recognition of one and two determinants on an antigen respectively. Science 169:1042.

28. Lafferty, K.J., and A.J. Cunningham. 1975. A new analysis of allogeneic interactions. Aust. J. Exp. Biol. Sci. 53:27.

29. Linsley, P.S., W. Brady, L. Groamaire, A. Aruffo, N.K. Damle, and J.A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. J. Exp. Med. 173:721.

30. Kündig, T.M., M.F. Bachmann, C. DiPaolo, J.J.L. Simard, M. Battegay, H. Lother, A. Gessner, K. Kühlke, P.S. Ohashi, H. Hengartner, and R.M. Zinkernagel. 1995. Fibroblasts as efficient antigen-presenting cells in lymphoid organs. Science 268:1343.

31. Xiang, Z.Q., and H.C.J. Ertl. 1994. A simple method to test the ability of individual viral proteins to induce immune responses. J. Virol. Methods 47:103.

32. Knight, S.C., J. Krejci, M. Malkovsky, V. Colizzi, A. Gautam, and G.L. 1985. The role of dendritic cells in the initiation of immune responses to contact sensitizers. I. In vivo exposure to antigen. Cell. Immunol. 94:427.

33. Gray, D. 1993. Immunological memory. Annu. Rev. Immunol. 11:49.

34. Sprent, J. 1994. T and B memory cells. Cell 76:315.

35. Wojczyk, B., S.H. Shakin-Eshleman, R.W. Doms, Z.Q. Xiang, H.C.J. Ertl, W.H. Wunner, and S.L. Spitalnik. 1995. Stable secretion of a soluble oligomeric form of rabies virus glycoprotein: influence of N-glycan processing on secretion. Biochemistry 34:2599.

36. Yakaukas, M.A., J.E. Marrow, S.E. Parker, A. Abai, G.H. Rhodes, V.J. Dwarki, and S.H. Gromkowski. 1993. Long-term anti-nucleoprotein cellular and humoral immunity is induced by intramuscular injection of plasmid DNA containing NP gene. DNA Cell. Biol. 12:771.

37. Donnelly, J.J., A. Friedman, D. Montgemery, J.W. Shiver, K.R. Leander, H. Perry, D. Martinez, J.B. Ulme, and M.A. Liu. 1994. Polynucleotide vaccination against influenza, P. 55. In E. Norby, F. Brown, R.M. Chanock, and H.S. Ginsberg (eds.): Vaccines 94. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

38. Xiang, Z.Q., B.B. Knowles, J.W. McCarrick, and H.C.J. Ertl. 1995. Immune effector mechanisms required for protection to rabies virus. Virology 214:398.

39. Danko, I., and J.A. Wolff. 1994. Direct gene transfer into muscle. Vaccine 12:1499.

40. Maul, G.G., A.M. Ishov, and R.D. Everett. 1995. Nuclear domain 10 (ND10) are the preferred sites where the nuclear replication cycle of herpes simplex virus type 1 begins. Virology 59:498–513.

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